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EPIGENETIC STUDIES IN ECOLOGY AND EVOLUTION

Evidence from pyrosequencing indicates that natural variation in animal personality is associated with DRD4 DNA methylation

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Abstract

Personality traits are heritable and respond to natural selection, but are at the same time influenced by the ontogenetic environment. Epigenetic effects, such as DNA methylation, have been proposed as a key mechanism to control personality variation. However, to date little is known about the contribution of epigenetic effects to natural variation in behaviour. Here, we show that great tit (*Parus major*) lines artificially selected for divergent exploratory behaviour for four generations differ in their DNA methylation levels at the dopamine receptor D4 (DRD4) gene. This D4 receptor is statistically associated with personality traits in both humans and nonhuman animals, including the great tit. Previous work in this songbird failed to detect functional genetic polymorphisms within DRD4 that could account for the gene–trait association. However, our observation supports the idea that DRD4 is functionally involved in exploratory behaviour but that its effects are mediated by DNA methylation. While the exact mechanism underlying the transgenerational consistency of DRD4 methylation remains to be elucidated, this study shows that epigenetic mechanisms are involved in shaping natural variation in personality traits. We outline how this first finding provides a basis for investigating the epigenetic contribution to personality traits in natural systems and its subsequent role for understanding the ecology and evolution of behavioural consistency.

Keywords: behaviour, birds, DNA methylation, epigenetics, personality

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Introduction

Personality—the general tendency of individuals to differ in patterns of behaviour that are consistent across time and over contexts—is important in explaining individual differences in health and fitness in both humans and nonhuman animals (Clark & Ehlinger 1987; Réale *et al.* 2007; John *et al.* 2010). A significant heritable component underlies part of the variation in personality traits (van Oers *et al.* 2005; van Oers & Mueller 2010); however, attempts to identify genetic polymorphisms consistently associated with personality traits have thus far met with limited success (van Oers & Mueller 2010; Balestri *et al.* 2014). Moreover, recent evidence shows that environmental experiences during early development can be just as important in explaining both variation and consistency in personality traits (Stamps & Groothuis 2010; Curley & Branchi 2013). It

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remains unclear, for both humans (Bouchard & McGue 2003) and nonhuman animals (van Oers & Mueller 2010), what proportion of personality results are fixed and what proportion is variable and plastic during an individual's lifetime. Therefore, it is important to understand the molecular mechanisms that link the heritable variation in animal personality traits to environmental developmental plasticity (Youngson & Whitelaw 2008).

Epigenetic mechanisms—the collective chemical and physical processes that programme the genome to express its genes in a time-, cell- and environment-dependent manner through nonmutagenic means (Jablonka & Raz 2009)—are good candidates to play an important role in explaining personality variation (Ledon-Rettig *et al.* 2013). These epigenetic modifications can be induced by rapid changes in the environment and are found to explain long-lasting developmental effects (Bossdorf *et al.* 2008) that may even pass across generations (van Oers & Mueller 2010; Groothuis & Trillmich 2011; Curley & Branchi 2013). Several molecular mechanisms have been discovered that are responsible for epigenetic influences on genome function, among which DNA methylation is one of the best studied (Jaenisch & Bird 2003; Bender 2004). Methylation of cytosines in CpG dinucleotide contexts, particularly within CpG islands (CGIs), can affect gene expression both in promotor regions (Bender 2004) and in the gene body (Ball *et al.* 2009).

An epigenetic basis for personality-related disorders has been recently suggested in humans (Kaminsky *et al.* 2008; Kumsta *et al.* 2013; Paquette & Marsit 2014), but in other animals only a handful of studies have investigated the link between variation in DNA methylation and behaviour, and these were mainly focused on domestic rodents in a laboratory setting (Weaver *et al.* 2004; Szyf *et al.* 2005; Champagne & Curley 2009; Herb *et al.* 2012; Massart *et al.* 2012; Dias & Ressler 2014). These studies on laboratory-bred animals have been critical for exposing the association of DNA methylation variation in candidate genes and the expression of these genes. For example in rats, variation in stress resilience was attributed to an epigenetically controlled transcription of the BDNF gene, resulting in differential expression. While low novelty-seeking rats were found to upregulate the expression of the BDNF gene following social defeat, no such reaction was present in high novelty-seeking rats (Duclot & Kabbaj 2013). Another study demonstrated that rats receiving more grooming as pups had lower stress responses and showed different methylation patterns of genes associated with the glucocorticoid stress response compared to rats that received less grooming (Weaver *et al.* 2004; Szyf *et al.* 2005). Experimental methyl supplementation altered both the epigenetic markings and the stress phenotype (Weaver 2005), indicating a causal epigenetic control of the plastic stress response. However, very little is known about an epigenetic involvement in explaining behavioural variation in behavioural traits in natural systems, and no information exists on the role of epigenetics in natural variation in personality (Ledon-Rettig *et al.* 2013). Studying the underlying epigenetic mechanisms such as DNA methylation in these ecological systems is essential to answer questions on its ecological and evolutionary significance.

In many studies on reward-seeking behaviours related to impulsivity, aggression, exploration and novelty seeking in humans and nonhuman animals, dopamine receptor D4 (DRD4) emerges as a major candidate gene explaining genetic variation (Ebstein *et al.* 1996; Dulawa *et al.* 1999; Szekely *et al.* 2004; Munafó *et al.* 2008; Flisikowski *et al.* 2009; Frieling *et al.* 2010). In great tits (*Parus major*), allelic variants in exon 3 of the DRD4 gene are statistically associated with exploratory behaviour in experimental populations that were selected for divergent levels of exploratory behaviour and in hand-reared wild birds (Fidler *et al.* 2007) and also in some (but not all) natural populations (Korsten *et al.* 2010; Mueller *et al.* 2013). However, the observed association could not be linked to any functional (nonsynonymous) single nucleotide polymorphism (SNP) or deletion site within the DRD4 gene or its flanking regions, although there were signs of selection for this polymorphism (Korsten *et al.* 2010; Mueller *et al.* 2013). Thus, DRD4 seems to play a role in heritable variation in great tit exploratory behaviour, but the underlying molecular mechanism remains to be demonstrated.

Here, we studied variation in methylation levels of the DRD4 gene in two great tit lines that were selected for high and low early exploratory behaviour for four generations and that showed heritable differences in exploratory behaviour. By investigating levels of DNA methylation in the DRD4 gene in brain and blood tissue of selection line birds, we showed that methylation variation at a CGI overlapping the DRD4 transcription start site is associated with heritable variation in exploratory behaviour. This suggests that heritable divergence in this behavioural trait may involve epigenetic modification of DRD4. This finding also suggests that to understand natural variation and evolutionary significance of personality variation in this ecological model system, future work should include efforts to understand the causes and consequences of DNA methylation variation at this candidate gene.

Materials and methods

Subjects

Birds originated from the fourth generation of two lines artificially selected for four generations for low (SE) and

high (FE) levels of early exploratory behaviour and were all born in 1997. Early exploratory behaviour is a combination of the fast or slow reaction towards a novel environment (exploratory behaviour) and a novel object (boldness), measured right after independence (for test protocol, see Drent *et al.* 2003). These personality traits have been validated in great tits and associated with several physiological, behavioural and life history traits and have also been found to influence fitness (van Oers & Naguib 2013). For the selection experiment, pairs of great tits were selected on high and low levels of exploratory behaviour and housed in half-open aviaries (2 × 2.5 × 4 m) to breed at the NIOO-KNAW. Eggs that were laid were transferred to a natural nest of foster parents when clutches were full. In the field, freshly hatched chicks were cross-fostered in such a way that each foster parent pair raised half a brood of FE and half a brood of SE chicks. When chicks were 10 days old, they were transferred back to the aviary facilities at the NIOO-KNAW and hand-reared until independence as described in Drent *et al.* (2003). From these offspring, we selected the most extreme birds for the next generation, avoiding inbreeding and multiple offspring per family. A heritability was found of 54% after fourth generation of selection (for test protocol see Drent *et al.* 2003).

We used both blood and brain samples from male and female great tits which died from natural causes in our aviaries (ages 5–8 years) and which were stored at −20 °C. Blood samples were collected while the birds were still alive in the period 2002–2005. For a sample overview, see Table S1 (Supporting information). Avian blood contains nucleated red blood cells, so more than 90% of the DNA isolated from avian blood samples originates from erythrocytes. We aimed to create a balanced sample set, but one blood sample failed in all tests; therefore, 12 SE birds (eight males and four females from five different families) and 11 FE birds (six males and five females from four families) were used to determine the DRD4 methylation levels in blood (Table S1, Supporting information). Brain tissue was available for a subset of birds for which we had blood samples: 10 SE (seven males and three females from five families) and 8 FE birds (five males and two females from four families; see Table S1, Supporting information).

DNA extraction

Blood samples consisted of either 10 μL whole blood in 1 mL Cell Lysis Solution (Gentra Puregene Kit, Qiagen, USA), or 10 μL of whole blood stored in Queen's buffer (Seutin *et al.* 1991). All samples were stored at room temperature. Total DNA was prepared using 250 μL of the stored blood samples with 750 μL Cell Lysis Solution (Gentra Puregene Kit; Qiagen) incubated with proteinase K at 55 °C overnight, followed by DNA extraction following the manufacturer's protocol. DNA was stored in DNA Hydration Solution (Qiagen).

Brain samples were collected from the same individual birds as used for the blood sample analysis (Table S1, Supporting information). Brains were dissected out of frozen birds (−20 °C), and hypothalamus- and hippocampus-enriched regions were isolated as described in Lindqvist *et al.* (2007). To ensure that we isolated DNA from the same area for each individual, we made landmarks on a cutting board to consistently cut the same part of the brains using a razor blade. These regions were then incubated overnight at 55 °C in 750 μL Cell Lysis Solution (Gentra Puregene Kit; Qiagen) with 20 μL proteinase K. About 250 μL of this lysed tissue was added to 250 μL Cell Lysis Solution. To remove excess of fat and proteins, 500 μL of 24:1 chloroform:isoamylalcohol was added and mixed until homogeneous, followed by 10-min centrifugation at 12 000 *g* after which the upper layer was collected. Cell Lysis Solution was added to this upper layer until 500 μL of sample liquid was obtained and total DNA was extracted according to the manufacturer's protocol. DNA was stored in DNA Hydration Solution (Qiagen), and the concentration was determined with a Nanodrop 2000 (Thermo Scientific, USA).

CpG position selection in the DRD4 gene for pyrosequence assay design

A CGI motif was searched in the DRD4 genomic region (GenBank Accession no. DQ006802) using CpGFinder (Softberry, USA) with the base pair numbering set to 1 on the transcription start site. Within the DRD4 gene, four regions were chosen that contained high densities of CpG dinucleotides within a short range and had enough surrounding sequence variety for primer design (Fig. 1). The DRD4 5′ upstream region was too dense with CpGs and primer design proved impossible. The PYROMARK ASSAY DESIGN SW 2.0 (Qiagen) was used to design the pyrosequence assays, which contain two PCR primers of which one is labelled with Biotin and one sequence primer opposite of the Biotinylated primer. The selection lines are still highly variable, and SNPs or deletions/insertions could lead to out-of-frame sequencing and were avoided as much as possible when developing the assays. If this proved impossible, the SNPs were added manually to the reference sequence. Assay A is located in exon 1, within the CGI, 241 bp downstream of the transcription start site, and contains 8 CpG sites within 73 bp of sequence length. This assay was only used for assessing the global

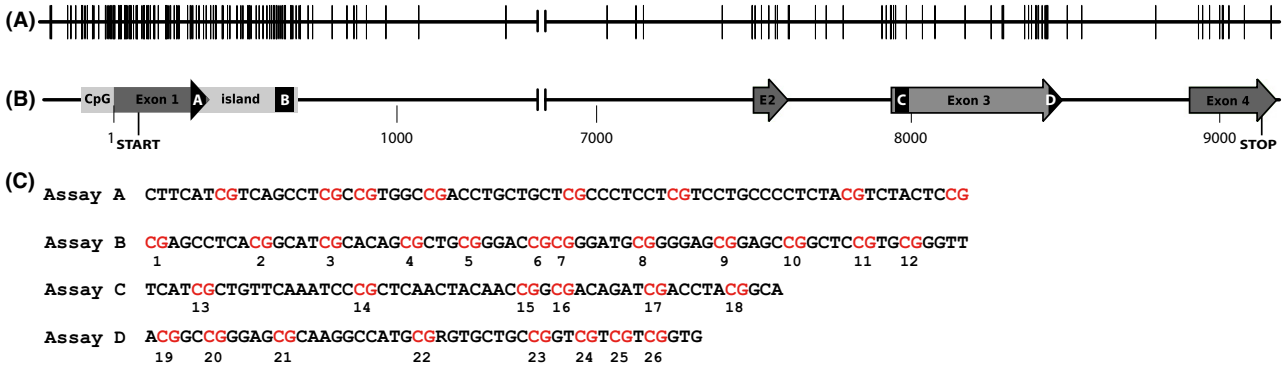

Fig. 1 Overview of the pyrosequence assay positions in the DRD4 gene. (A) CpG density in the DRD4 gene. (B) Structure of the DRD4 gene with the pyrosequence assay positions (black box with white letters), exons (dark grey arrows) and CpG island (light grey box). The numbers below the axis denote base pairs starting from the transcription start site. (C) Target sequence of the four different assays with in red the CpG sites, which are numbered consecutively across assays B, C and D.

methylation level in exon 1, because many samples were excluded due to unreliable methylation scores, resulting in a too low sample size to be analysed for sex and personality differences. Assay B is located in intron 1, within the CGI, 515 bp downstream of the transcription start site, and contains 12 CpG sites within 71 bp of sequence length. Assay C is located in exon 3, 7918 bp downstream of the transcription start site, and contains six CpG sites within 55 bp of sequence length. Assay D is located in exon 3, 8423 bp downstream of the transcription start site, and contains eight CpG sites within 47 bp of sequence length. See Table S2 (Supporting information) for an overview of the primer sequences.

Bisulphite conversion, methylation-specific PCR and pyrosequencing

One or 2 μg of total genomic DNA was bisulphite-converted using the EpiTect bisulphite conversion kit (Qiagen) and eluted from the supplied washing column with 20 μL of supplied elution buffer. One microlitre of this eluate was used in a 25-μL PCR using the PyroMark PCR Kit (Qiagen) according to the manufacturer's protocol but without Q-solution, 100 nM or 200 nM forward primer and 100 nM or 200 nM reverse biotinylated primer (see Table S2 for primer overview, Supporting information). PCR conditions were as follows: 94 °C for 15 min. followed by 45 cycles of 94 °C for 30 s, 61 °C for 30 s, 72 °C for 30 s; ending with 72 °C for 7 min. The PCR product was purified using streptavidin Sepharose HP beads (GE Healthcare Europe, the Netherlands) followed by hybridization of the sequencing primer with the biotinylated PCR product as described in the Pyromark Q24 vacuum workstation guide (Qiagen). The methylation percentage per CpG position was determined using the PYROMARK Q24 ADVANCE software (Qiagen). In short, pyrosequencing produces quantitative measures of DNA methylation based on a sequencing-by-synthesis method (Tost & Gut 2007). The DNA methylation percentage is assessed by the ratio of real-time incorporated C and T nucleotides, through the conversion of released pyrophosphate into a light signal. In assays B, C and D, we included a control step to determine the completeness of bisulphite conversion (Table S2, Supporting information). We sequenced each individual multiple times for each assay. The PYROMARK Q24 ADVANCE software checked reliability of the results, and samples containing CpG sites marked as unreliable methylation results by the software were rerun. CpG sites that were again marked as unreliable after rerunning were excluded. After this quality control, we averaged the methylation level for each site per individual for all reliable samples (range 1–4) for further analysis. The intra-assay coefficient of variation (CV) was 1.8% and the interassay CV was 5.4%.

Verification of pyrosequence results with bisulphite sequencing

To verify that no mutation or SNPs could interfere with the pyrosequencing of assay B, which showed the most interesting variation in our study, the PCR products were cloned and sequenced (Macrogen, the Netherlands). The PCR products were generated with primer set AssayB_Fw and AssayB_Rv (without biotin label) from each individual sample and were pooled prior to sequencing. This resulted in sequences from 33 clones containing the PCR fragment from assay B (Fig. S1, Supporting information). These sequences indicated that no previously unknown SNP or other mutation could have interfered with the pyrosequencing and that the results in our study are not due to out-of-frame sequencing. In addition, no parental imprinting was observed as this would have been observed as

fragments with none to low methylation and fully methylated fragments (Fig. S2, Supporting information).

Statistical analysis

To investigate possible sex- and personality-dependent differences in patterns of DNA methylation in blood and brain samples, a joint analysis including all samples of both tissue types was performed using a general linear mixed model (GLMM) with the percentage of methylation as dependent variable and personality type (line; FE or SE), tissue (brain or blood), site (CpG site) and sex (male, female) and their two- and three-way interactions as fixed factors. Family (nested within personality type) and individual (nested within family and within personality type) were included in the model as random factors to account for the fact that individuals within families were not independent measures due to the hierarchical structure in our data.

To assess associations between methylation levels and sex and personality in the two tissues separately, we also performed GLMMs per tissue type (blood and brain), including site, sex and personality type and their two- and three-way interactions. We conducted a backwards elimination method and provide F and *P* values for the factors before removing them from the model. For significant interactions, we conducted pairwise post hoc comparisons (with *F*-tests and DF between 14.5 and 16.0) between the estimated marginal means with Bonferroni adjustments for multiple comparisons using the Pairwise Comparisons options in the linear mixed model analysis.

In some instances, no reliable pyrosequence results were obtained for a particular sample and those samples were thus excluded from the analysis. Therefore, the number of blood tissue samples analysed for site 1–12 (assay B) was 11 SE and 9 FE; for site 13–18 (assay C), 7 SE and 10 FE; and for site 19–26 (assay D), 8 SE and 8 FE. The number of brain tissue samples analysed for site 1–12 (assay B) was 9 SE and 8 FE; for site 13–18 (assay C), 8SE and 6FE; and for site 19–26 (assay D), 10 SE and 8 FE. For all models, the residuals were checked for normality. We used IBM SPSS Statistics 22 for Windows for all statistical analyses.

Results

DRD4 methylation pattern

We found one CGI ranging from −118 bp to 632 bp, overlapping part of the putative promotor region, the transcription start site, the translation initiation site and the 5′ part of intron 1 (Fig. 1). This CGI contained a CG percentage of 73.5% and an observed to expected CpG ratio of 0.895. In mammals and chicken, approximately 50–65% of transcription starts sites (TSS) overlap with CGI predictions (Li *et al.* 2011; Hu *et al.* 2013; Long *et al.* 2013), which is seen here for DRD4 too. However, the CGI in the great tit DRD4 shows features of both a TSS CGI but also gene body CGI as it overlaps TSS, exon 1 and part of intron 1.

The methylation levels in the DRD4 gene were assayed in four regions of approximately 60–80 base pairs that contained a high density of CpG dinucleotides. We first assessed mean methylation levels per assay in blood samples for both personality groups together and observed that DRD4 methylation levels are low in assay A (7.4% methylation) located in exon 1, which corroborates observations in mammals and chicken that most CGIs overlap with regions of low methylation (nonmethylated islands) (Long *et al.* 2013). The methylation levels increase sharply in assay C (89.6%) and D (90.8%), located in the 5′ and 3′ region of exon 3, respectively, while assay B (35.2%) located in intron 1 shows intermediate methylation levels (see Fig. 2). This increase in DNA methylation towards the 3′ end of the gene is consistent with patterns of gene body methylation in mammals, where DNA methylation level of the first exon is tightly linked to transcriptional silencing (Brenet *et al.* 2011). The low methylation in assay A may suggest that DRD4 is (highly) expressed in great tits. The strong increase in DRD4 methylation towards 3′ could be an additional indication for high expression level (but see Ball *et al.* 2009; Brenet *et al.* 2011).

Methylation levels associate with personality type

Next, we determined methylation levels for every CpG site within assays B, C and D for each sample individually. In an initial analyses, we found that differences in

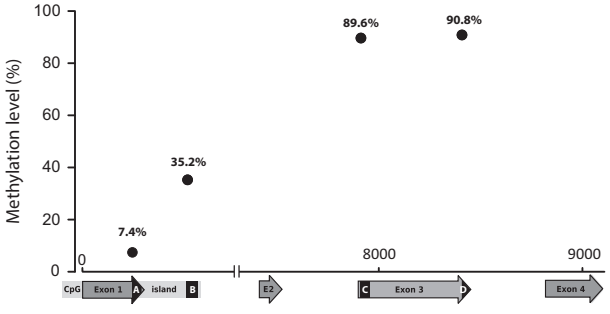

Fig. 2 The mean methylation levels in blood tissue. The increase in methylation towards the 3′ end of DRD4 is shown with the position of the assay in the gene on the *x*-axis. The four pyrosequence assay positions (A, B, C and D; black box with white letters), exons (dark grey arrows) and CpG island (light grey box) are shown on the *x*-axis with the numbers denoting base pairs starting from the transcription start site.

Table 1 Model output from a combined brain and blood analysis on methylation levels

Factor	*F*	*P*
Ptype*Site*Sex	$F_{25,699.3} = 0.60$	0.94
Tissue*Site*Sex	$F_{25,724.1} = 0.99$	0.48
Ptype*Tissue*Site	$F_{25,749.9} = 1.56$	0.04

A general linear mixed effect model (GLMM) was used to test sex- and personality-related differences in methylation levels between the blood and brain tissue. Personality type (PTYPE), sex, CpG position (site) and tissue (blood or brain) were included as fixed factors. Family (nested within personality type) and individual (nested within family and within personality type) were included in the model as random factors to account for the hierarchical structure in our data. Only the three-way interactions are presented, but they were tested with the two-way interactions and main effects in the model (not shown).

methylation levels per CpG site between personality types were tissue dependent (Table 1; Ptype*site*tissue, $F_{25,749.9} = 1.56$, $P = 0.04$). We therefore analysed results for both tissues separately, even though in general we found a high correlation ($r_p = 0.97$, $P < 0.0001$) between blood and brain methylation levels.

CpG methylation levels in blood tissue differed significantly between FE and SE individuals, with FE showing higher methylation levels in some but not all sites (Fig. 3A and Table 2; Ptype*site, $F_{25,371.0} = 8.78$, $P < 0.0001$). A post hoc analysis revealed that these significant differences were restricted to CpG sites 1–11 and showed a tendency for position 12 (assay B; $P = 0.059$; Fig. 3A) while for position 13–26 (assays C and D) methylation levels did not differ significantly between individuals originating from the two selection lines. Similarly, the CpG methylation levels in brain tissue were different between FE and SE and this effect differed between sites as well (Table 2; Ptype*site, $F_{25,364.1} = 2.70$, $P < 0.0001$). A post hoc analysis testing the difference between the lines at each site separately revealed that also in brain tissue, FE individuals had higher CpG methylation and that this was caused by differences at CpG positions 1–6, 8 and 9 (assay B), while FE and SE individuals did not differ in CpG methylation for other positions (Table 2 and Fig. 3B).

Sex-specific differences in methylation levels

In addition to the observed differences in personality type, sexes also differed in methylation levels. Although sex differences in CpG methylation levels were not tissue dependent (sex*tissue, $F_{25,724.1} = 0.99$, $P = 0.48$), the sex effect could only be shown in blood ($F_{25,371.1} = 2.54$, $P < 0.001$; Table 2) and not in brain tissue ($F_{25,339.3} = 0.26$, $P = 0.16$; Table 2). In blood, methylation levels were significantly higher for females compared with males for CpG sites 1–6, and the difference showed a trend for CpG sites 8 and 9 (Table 2). This effect of sex was the same for both selection lines (sex*Ptype, $F_{25,346.1} = 0.78$, $P = 0.77$).

Discussion

To explore a possible epigenetic contribution to personality trait variation, we set out to study the levels of DNA methylation at the DRD4 gene in great tits artificially selected for contrasting levels of early exploratory behaviour (FE vs. SE) during four generations. We found significant methylation differences between FE and SE exploring birds in the downstream part of a CGI that partly overlaps the gene's first intron and transcription start site. This suggests that epigenetic variation at DRD4 is involved in functional, heritable divergence in great tit exploratory behaviour. Ideally, we would have associated DRD4 methylation levels in blood and brain tissue with the expression of DRD4 in these tissues. Unfortunately, we were unable to study the brain expression levels of DRD4 in the samples studies, because these selection line birds were not sacrificed for the purpose of gene expression analysis and died of natural causes. Thus, the functional link between DRD4 methylation and expression remains to be demonstrated in future gene expression studies.

DRD4 has been implicated in personality trait variation in different species before. A study on repetitive variants in DRD4 exon 3 in humans showed a positive correlation between DRD4 genotypes and novelty seeking (Ebstein *et al.* 1996), and a more recent meta-analysis showed that this association reflects a more general association between DRD4 and novelty-seeking behaviour (Munafó *et al.* 2008). Similarly, in birds, high levels of genomic variation are present in functional regions of the DRD4 gene (Abe *et al.* 2011). Previous studies in great tits have also found genetic associations of DRD4 and personality differences; however, pinpointing functional genetic DRD4 variation has remained elusive as statistically significant DRD4-personality associations were based on a synonymous (and thus noncausal) SNP (Fidler *et al.* 2007; Korsten *et al.* 2010). One possible explanation for the presence of a statistical association but the lack of local functional genetic variation is allele-specific epigenetic differences that are associated with SNP variation in DRD4, as has been observed previously in humans (Docherty *et al.* 2012). Alternatively, functional epigenetic variation that is not associated with SNP variants may account for missing heritability (Maher 2008), and might be partly responsible for recent failure in a genomewide

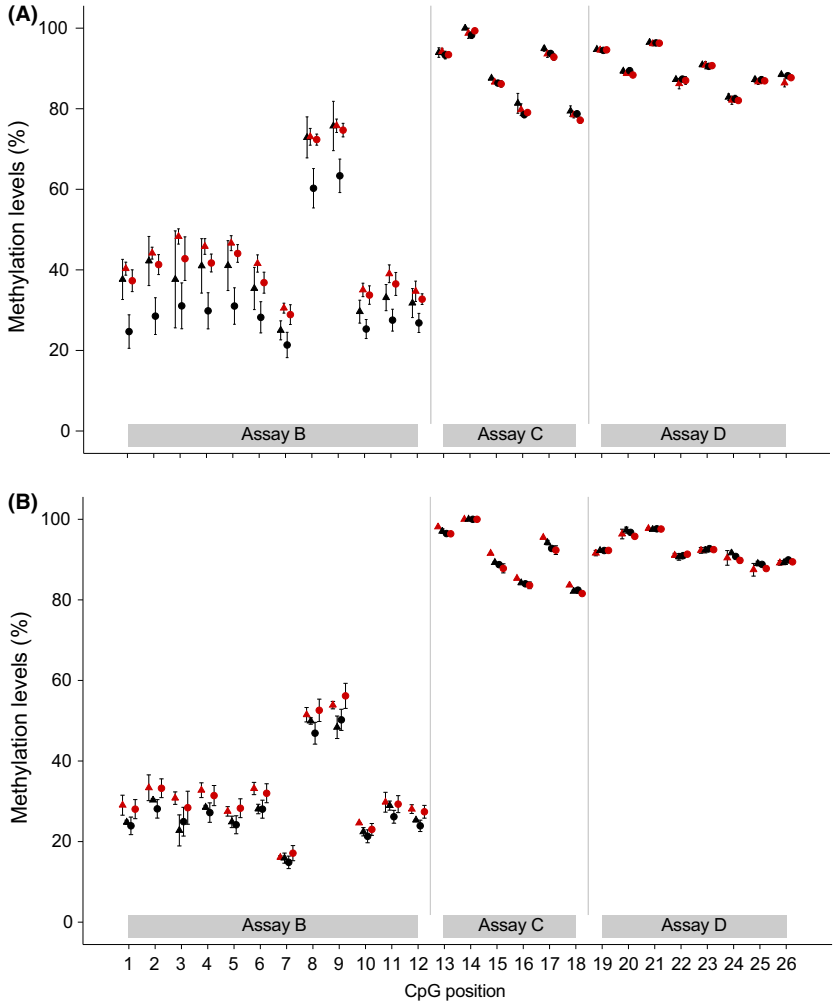

Fig. 3 DNA methylation levels in blood and brain tissue. Methylation levels in (A) blood tissue and (B) brain tissue for assays B, C and D, per selection line and sex, where triangles indicate females and circles indicate males. Black markers indicate slow selection line (SE), and red markers indicate fast selection line (FE). Error bars represent SEM.

association study to detect a significant association with personality traits for several great tit populations (Mueller *et al.* 2013).

Although the link between DNA methylation and gene expression is not always straightforward (Jones 2012), we hypothesize that differential methylation at the DRD4 gene affects gene activity and ultimately personality variation. Indirect evidence for a functional interpretation is provided by a recent great tit study using whole-genome bisulphite and RNAseq data. This study showed that across all genes, higher CG methylation at transcription start sites and within gene bodies is associated with lower gene expression in the great tit (Fig. 4b, Laine *et al.* 2016). This indirectly supports the interpretation that higher DNA methylation in the 5′ region of a gene (as we observe for DRD4) is associated with reduced gene expression.

We also point out that the pattern of DRD4 methylation that we observed is consistent with a functional role: the methylation difference between selection lines was not constant across the entire length of DRD4 but was restricted to an area near the transcription start site, whereas no significant methylation differences were found in two highly methylated regions in exon 3 (assays C and D). The methylation levels of around 35% observed in the differentially methylated region of assay B correspond to low-methylated regions (LMRs) observed in mice (Stadler *et al.* 2011). These LMRs are often associated with enhancer regions and show dynamic methylation, owing either to competing methylation and demethylation in time or to inaccurate maintenance of methylation during cell division (Stadler *et al.* 2011; Jones 2012). Only the first intron (assay B) showed high variation in methylation levels, indicating

Table 2 Model output for separate analysis on blood and brain tissues

	Factor	*F*	*P*
Blood	Ptype × Site × Sex	$F_{25,346.1} = 0.78$	0.77
	Ptype × Sex	$F_{1,14.2} = 0.85$	0.37
	Site × Sex	$F_{25,371.1} = 2.54$	<0.0001
	Post hoc analysis	Site 8	0.052
		Site 9	0.059
		Site 10–26	>0.10
	Ptype × Site	$F_{25,371.0} = 8.78$	<0.0001
	Post hoc analysis	Site 1–11	<0.05
		Site 12	0.059
		Site 13–26	>0.10
Brain	Ptype × Site × Sex	$F_{25,314.23} = 0.21$	1.00
	Ptype × Sex	$F_{1,11.5} = 2.28$	0.16
	Site × Sex	$F_{25,339.3} = 0.26$	1.00
	Ptype × Site	$F_{25,364.1} = 2.70$	<0.0001
	Post hoc analysis	Site 1–6,8,9	<0.05
		Site 12	0.09
		Site 7,13–26	>0.15
	Sex	$F_{1,11.2} = 0.14$	0.72

Separate general linear mixed model (GLMMs) per tissue type (blood and brain) were performed to assess associations between methylation levels and sex and personality over the different CpG positions. Personality type (PTYPE), sex, CpG position (site) and tissue (blood or brain), their two-way interactions and the three-way interaction between personality type, sex and CpG position were included as fixed factors. Family (nested within personality type) and individual (nested within family and within personality type) were included in the model as random factors to account for the hierarchical structure in our data. We conducted a backwards elimination method and provide F and *P* values for the factors before removing them from the model. We conducted pairwise post hoc comparisons with Bonferroni adjustments for multiple comparisons of the significant interactions site*sex and Ptype*site in blood and Ptype*site in brain. The main effect of sex in blood is not shown, due to the site*sex interaction. The number of blood tissue samples analysed for site 1–12 was 11 SE and 9 FE; for site 13–18, 7 SE and 10 FE; and for site 19–26, 8 SE and 8 FE. The number of brain tissue samples analysed for site 1–12 was 9 SE and 8 FE; for site 13–18, 8 SE and 6 FE; and for site 19–26, 10 SE and 8 FE.

that methylation in this region might be in a dynamic state, which suggests the presence of putative DRD4 enhancers in this region. Differential methylation at intronic enhancers is known to affect gene expression (Unoki & Nakamura 2003; Hoivik *et al.* 2011), and DRD4 activity may thus be affected, contributing to observed variation in early exploratory behaviour.

One limitation of our study is that we have screened DNA methylation in DRD4 only and not in other genes. Although DNA methylation levels can covary considerably across the genome causing consistent differences in methylation levels between samples at many loci (Shabalin *et al.* 2015), individual differences in methylation levels are likely to vary among genes. It is therefore important in future work to demonstrate whether the observed methylation differentiation between selection lines is specific to DRD4 or if it is a present over larger genomic scales. In our study, we did not screen separate control genes for this purpose; however, we used two 3′ regions within the DRD4 gene that serve as suitable controls (assays C and D). These control regions are towards the 3′ end of the gene and show no difference in methylation between the selection lines. Instead, we only find methylation differences between the lines in assay B, which is towards the 5′ end of the gene. Thus, while validating more genes and genomic loci would be desirable, it is highly unlikely that the observed methylation differences between selection lines merely reflect consistent differences in methylation levels over large genomic regions.

The observed methylation difference between fast and slow exploring birds provides supporting evidence for the involvement of DRD4 in determining variation in early exploratory behaviour. Moreover, the results suggest that to understand the underlying mechanisms of natural variation and evolutionary divergence in great tit personality traits, it may be important to further explore the causes and consequences of DRD4 methylation. Recently, methylation levels in DRD4 and serotonin transporter (SERT) were found to be higher in urban compared with forest-dwelling great tits (Riyahi *et al.* 2015). No clear association between DNA methylation and exploratory behaviour was found in that study for DRD4 or SERT. To our knowledge, our study therefore presents the first empirical evidence for the involvement of epigenetic mechanisms in personality trait divergence in a wild species.

This first finding, in a species that has become an ecological model species for natural behavioural studies (van Oers & Naguib 2013), can help to focus new lines of investigation on the ecological and evolutionary epigenetics of animal behaviour. For instance, one intriguing question concerns the heritability of DRD4 methylation variation. The observed DRD4 methylation differences between selection lines suggest transgenerational consistency of DRD4 methylation that is associated with exploratory behaviour. This is in contrast with a study comparing DRD4 methylation patterns in human monozygotic and dizygotic twins, where no heritable effect but only familiar effects were found (Wong *et al.* 2010). Unlike plants, in which meiotic stability of cytosine methylation is well established (Cortijo *et al.* 2014), transgenerational consistency of methylation patterns in vertebrates may involve (i) repeated de novo establishment of the same methylation pattern in each new generation after widespread DNA methylation

resetting between generations (Feng *et al.* 2010) controlled by underlying genetic differences, (ii) transmission of meiotically stable epi-alleles to the next generation without resetting (Manikkam *et al.* 2012) or (iii) parent–offspring behavioural interactions that are perpetuated each generation (Weaver *et al.* 2004). Our experimental design, in which individuals from both selection lines are cross-fostered and reared in similar environments, rules out parent–offspring interaction and environmental induction as a cause of consistent methylation differences between the selection lines and we assume a widespread DNA methylation resetting between the generations that is under genetic control. As no cis-genetic variation at DRD4 was observed (Docherty *et al.* 2012), the establishment of line-specific DRD4 methylation is likely under trans-genetic control. Identifying such trans-acting loci that control DRD4 methylation, for instance using QTL approaches based on between-line crosses, may be an important step in understanding the mechanistic basis of divergence and adaptation in personality traits.

Another important question is to what extent environmental effects can modify DRD4 methylation. Personality traits typically show long-term consistency within individuals, still there is adaptive significance in a considerable degree of plasticity in response to early developmental conditions (Groothuis & Trillmich 2011). A popular idea is that through environmental effects on DNA methylation, behavioural phenotypes can be controlled in concert with environmental demands (Kappeler & Meaney 2010; Jensen 2013), and may be effectuated via maternal effects, via for example maternal hormones, during embryonic development. The case of DRD4 in great tits, whose methylation may be partly under trans-genetic control, may offer an opportunity to investigate whether and how genetic and environmental control of DRD4 methylation may be balanced to jointly determine the behavioural phenotype.

In this study, we show that epigenetic patterns in DRD4 are associated with personality difference in great tits, indicating a functional role for DRD4 in explaining personality variation. In addition, the epigenetic expression regulation is partly heritable, indicating that genetic factors may play a role in the observed methylation variation. This might explain the dynamic regulation of personality, as the observed epigenetic variation makes the system flexible in a consistent but plastic way.

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E.C.V., A.C.M. and M.V.Z performed the research. E.C.V. analysed data. S.P.C. dissected the brains. K.J.F.V. and Kv.O. designed the research and analysed data. K.v.O. provided the bird samples. E.C.V., K.J.F.V. and K.v.O. wrote the manuscript. All authors provided input during the writing of the manuscript.

Data accessibility

Genotype and phenotype data were deposited in the Dryad repository: doi: 10.5061/dryad.fj662.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Samples of birds used in this study.

Table S2 Primer and target sequences of the four pyrosequence assays.

Fig. S1 Alignment of assay 5 PCR fragments showing the absence of unknown SNPs.

Fig. S2 Diagram showing the CpG methylation of the complete region amplified by the assay B primer set.